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Clinical Studies of COMET for Total LSCD: a Review of the Methods and Molecular Markers for Follow-Up Characterizations

Eustachio Attico [1] · Giulia Galaverni [1] · Graziella Pellegrini [1,2]

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Abstract

Purpose of Review This review outlines the main features of the clinical trials where cultivated oral mucosa epithelial cell transplantation (COMET) was performed, aiming to underscore a link between the clinical outcome and the expression of specific markers during the follow-up of patients, characteristic for a defined epithelium (cornea, oral mucosa, or conjunctiva) or related to vascularization.

Recent Findings Currently, little is known about the reasons underlying the success or failure of COMET. To address this issue, we focused on tissue characterization at the molecular level, highlighting the findings concerning angiogenesis.

Summary There are several discrepancies in the outcomes of COMET clinical trials. While some corneal/conjunctival markers can be considered reliable for understanding the biological mechanisms that drive corneal repair after transplants, a unique marker specifically expressed in the oral mucosa and an accurate study of the vascularization processes are currently missing. Together, these insights will help forecast successes and failures of these technologies.

Keywords Oral mucosa · Cornea · Cultivated oral mucosa epithelial cell transplantation (COMET) · Cultivated autologous oral mucosal epithelial cell sheet (CAOMECS) · Total bilateral limbal stem cell deficiency (LSCD) · Biomarkers

Introduction

The human cornea, which is crucial for vision, is also an essential protective barrier against external pathogens. It is developed and supported by limbal epithelial stem cells (LESCs), the corneal progenitor cells that are situated in the limbus, which constitutes a transitional ring-shaped zone between the cornea and conjunctiva. In its basal layers, the limbus hosts the so-called palisades of Vogts, which are optimal niche for LESCs. The palisades of Vogts comprise melanocytes, stromal fibroblasts, immune cells, and other epithelial non-stem cells that support the progenitor cells [1, 2].

Normally, LESCs are quiescent, but through a streamlined process, become competent for the renewal of the corneal epithelium, resulting in the migration and centripetal differentiation from the limbus towards the center of the eye. In case of corneal injury, LESCs are rapidly activated to bring about tissue repair. Damage to LESCs owing to injuries or pathologic status may result in a condition defined as limbal stem cell deficiency (LSCD). In this case, the priority is to close the wound and prevent the entry of pathogens; thus, conjunctival stem cells perform these protective functions by migrating over the cornea, undergoing a process known as "conjunctivalization," to form the so-called *pannus* [3]. The presence of conjunctival cells induces the formation of blood vessels (neovascularization), resulting in opacity, loss of vision, pain, and other symptoms.

LSCD can be defined as "total," when LESCs are completely lost and the entire cornea undergoes conjunctivalization, or "partial," when at least a part of the limbus is spared and the conjunctivalization is not complete.

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✉ Eustachio Attico
eustachio.attico@unimore.it

Giulia Galaverni
giulia.galaverni@unimore.it

Graziella Pellegrini
graziella.pellegrini@unimore.it

[1] Interdepartmental Centre for Regenerative Medicine "Stefano Ferrari", University of Modena and Reggio Emilia, Via Gottardi 100, Modena, Italy

[2] Holostem Terapie Avanzate, Via Gottardi 100, Modena, Italy

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Moreover, LSCD can be bilateral, involving both eyes, or unilateral, when only one eye is affected [4••].

During the past 30 years, many therapeutic approaches have been tested. A technique was developed in 1989 by Kenyon and Tseng, which was a milestone. Using this technique, conjunctivo-limbal autograft transplantation (CLAU) consisting of two large biopsies comprising limbus and conjunctiva from the uninjured eye (or part of the healthy eye in case of partial LSCD) to the completely injured eye was achieved [5].

For total bilateral LSCD, Tsai and Tseng developed a process known as keratolimbal allografting (KLAL), where the conjunctivo-limbal tissue is harvested from a living related donor or cadaver. Immunosuppression is required to prevent the rejection of the graft [6].

In 1997, Pellegrini et al. developed an ex vivo amplification protocol known as cultured limbal stem cell transplantation (CLET). This technique entails obtaining a small biopsy, measuring about 2 mm^2, from a healthy spared zone of the limbus of the patient's eye, expanding the corneal stem cells in vitro over a fibrin scaffold and transplanting them over the injured eye after removing the conjunctival *pannus* [7]. An approach that avoids the culturing step, based on the principle of CLET, is a simple limbal epithelial transplantation (SLET), a surgical procedure consisting of harvesting a 4 mm^2 biopsy from the healthy limbus of the eye, cutting it in small pieces, and placing them directly over an amniotic membrane disposed onto the injured cornea [8]. These limbal fragments behave like small explants when cultured directly in vivo on the cornea of the patients.

However, these methods are not applicable in total bilateral LSCD, owing to the complete absence of healthy limbus in both eyes; therefore, allogenic CLET (implicating all the related issues of allogeneic transplantation mentioned before) or other autologous sources of stem cells, such as conjunctival stem cells, mesenchymal stem cells (MSCs), human embryonic stem cells (hESCs), and induced pluripotent stem cells (iPS), have been explored [9–11]. Yet, the only autologous non-limbal cells that have successfully been used in a clinical setting are stem cells of the oral mucosa [12]. The procedure, named as "cultivated oral mucosa epithelial transplantation" (COMET), has been widely used in the last 20 years (Fig. 1a) with a success rate of approximately 70% [13••]. The oral mucosa is suitable because of its high regenerative potential, easy and multiple accesses for biopsy collection, and absence of keratinized epithelium. A different oral mucosa cell-based method is the CAOMECS (cultivated autologous oral mucosal epithelial cell sheet), where cells are seeded on a temperature-sensitive support in order to transplant the cell sheet without any additional scaffold [14, 15].

The success of COMET can be explained by two mechanisms of action: "engraftment" or "stimulation" [16, 17]. In engraftment, when LESCs are deficient ("anatomical deficiency"), in vitro oral mucosa tissue grafts containing stem cells are placed over the cornea of the patient to regenerate the epithelium lifelong. Alternatively, the stimulation mechanism means that few residual LSCs remained in the injured cornea, but in a hostile inflammatory environment are unable to regenerate the corneal epithelium ("functional deficiency" of LSCs). When an oral mucosa graft substitutes the pathologic *pannus*, the remaining LSCs are biologically stimulated and they slowly renew to regenerate an autologous cornea. This scenario has been observed to be possible in allograft limbal transplantation [18, 19]. A mixed pattern based on the two discussed mechanisms is also possible (Fig. 1b). To understand the biological processes underlying COMET, biomarkers, such as proteins, have been used to phenotypically characterize post-surgery corneas [12, 20].

Each study or clinical trial has specific technical approaches to prepare COMET grafts. A number of reviews that have focused on COMET have thoroughly analyzed the literature on culture methods, surgery, substrates/carriers, and many other aspects [13, 21, 22].

In this review, we focus on the molecular tissue characterization performed after COMET (or CAOMECS) clinical trials and on the molecular evaluations concerning angiogenesis.

Methods of Literature Search

We searched the PUBMED database using the following search terms: limbal stem cell deficiency, oral mucosa, and transplantation. The search was filtered to display trials involving humans; only clinical trials or case reports were considered. For a more exhaustive analysis, these PUBMED results were integrated with a few articles extracted from a review recently written by Cabral et al. [13••].

A total of 27 articles were analyzed, spanning the years 2004 to 2020.

LSCD Etiology and Diagnosis

Etiology

Among the acquired causes that accounted for the majority of LSCD cases, corneal burns were, by far, the most common etiology (156/360 eyes, 43.3%), with chemical injures as the leading cause (110/360 eyes; 30.42%), followed by thermal damage (46/360 eyes; 12.91%).

Stevens–Johnson syndrome, together with toxic epidermal necrolysis, is the second leading cause of LSCD (87/360 eyes, 24.17%), followed by ocular cicatricial pemphigoid (54/360 eyes; 15%) and other conditions such as keratitis, Lyell syndrome, trachoma, contact lens wear, hepatitis C, squamous cell carcinoma, graft-versus-host disease, radiation, and drug toxicity.

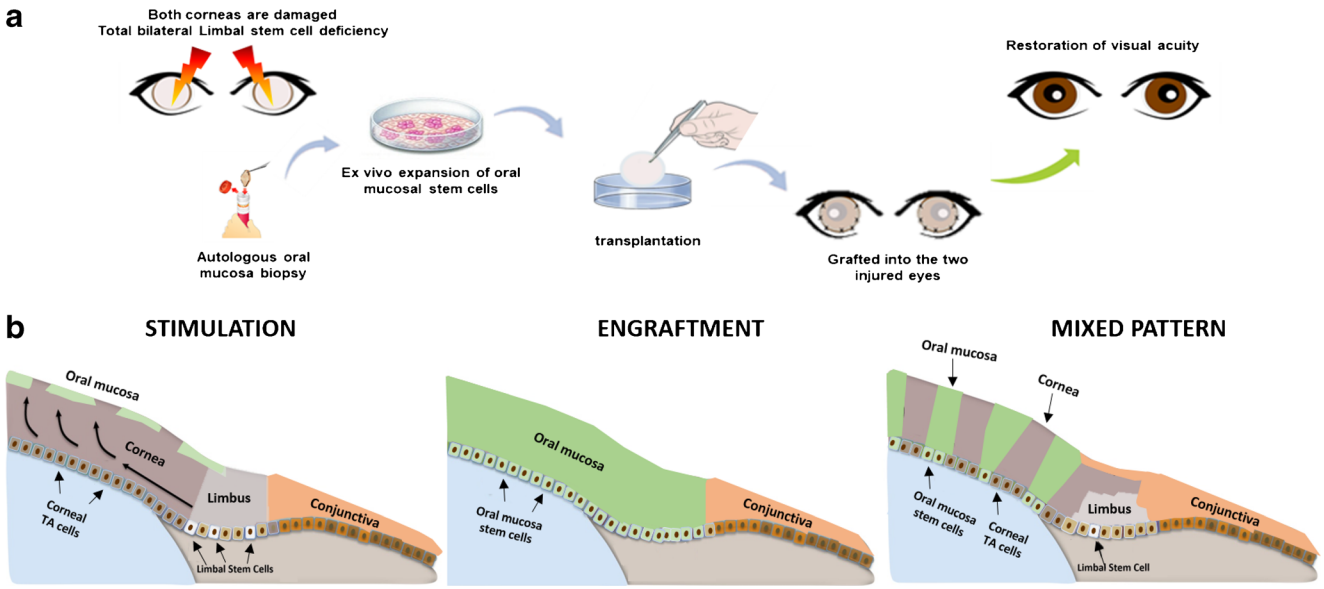

Fig. 1 Clinical procedure and conceivable outcomes subsequent to cultivated autologous oral mucosa transplantation (COMET) onto the ocular surface. **a** Critical steps involved in the production of autologous oral mucosa cells for corneal repair after bilateral damages. **b** Overview of the possible mechanisms of actions following the COMET procedure. TA, transit-amplifying

Among the hereditary causes, aniridia is the most common condition in individuals with LSCD (23/360 eyes; 6.39%), followed by diseases such as severe Groenouw dystrophy, Salzmann's corneal degeneration, and cystinosis (Fig. 2).

Worth of note, in the etiology analysis, some data could be redundant, since few patients were analyzed at different time points, and thus included in more than one clinical trial.

Overall, these findings are very similar to those reported by Cabral et al. in a list of 24 clinical trials [13••].

Diagnosis

The diagnosis of LSCD is primarily based on clinical signs, such as loss of normal limbal architecture (especially of palisades of Vogt) and thin and irregular corneal epithelium, which is progressively invaded by new blood vessels and cells of conjunctival epithelium migrating from the edges. Thus, patients usually present with corneal opacity, formation of a fibrovascular *pannus*, stromal scarring, and keratinization [4].

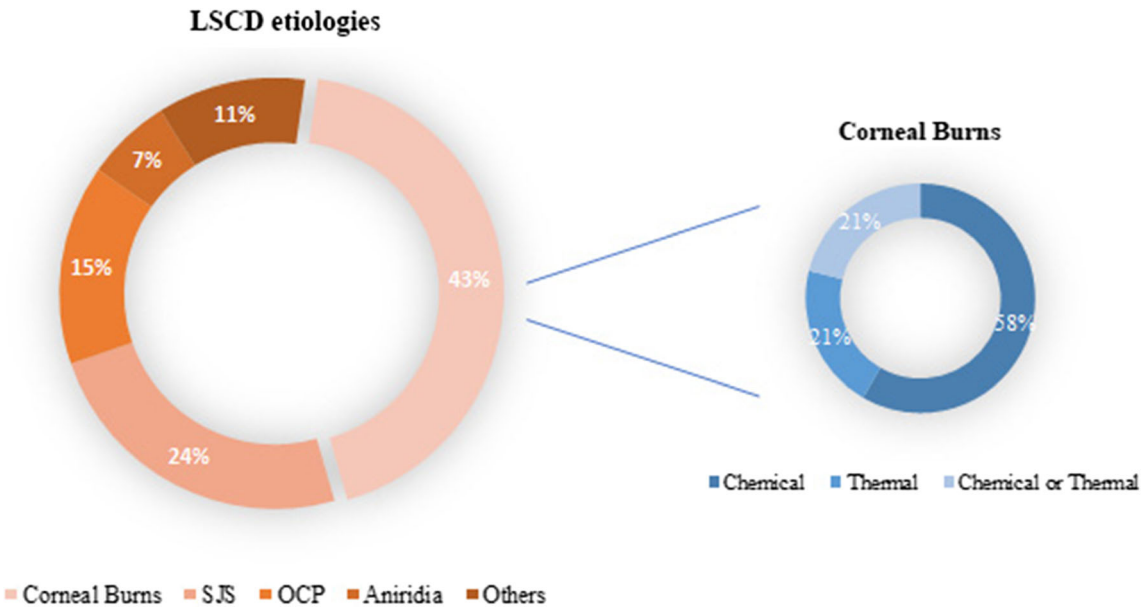

Fig. 2 Etiologies of LSCD patients involved in the examined clinical studies. SJS, Stevens–Johnson syndrome; OCP, ocular cicatricial pemphigoid

The diagnosis is usually supported by irregular fluorescein staining, which highlights the pattern and distribution of epithelial defects. These clinical features can be generally confirmed using standard laboratory tests, such as impression cytology (IC), which allows for the investigation of markers, primarily cytokeratins (CK or K) and mucins (MUC), which are expressed in the most superficial layers of the corneal and conjunctival epithelium (respectively K3, K12 and K19, K7, K13, MUC5AC) [23]. In vivo confocal microscopy (IVCM) is useful in evaluating and quantifying the described microstructural changes in the corneal and limbal epithelia, while emerging techniques, such as anterior segment optical coherence tomography (AS-OCT), represent a more convenient method of imaging and measuring epithelial thickness, *pannus* depth, and limbal crypts [24].

Culture Protocols in Clinical Trials

Biopsy

The culture protocol used for COMET in the 27 clinical studies that are analyzed in this study differs considerably in many aspects [13, 21], including the site from which mucosal tissue was harvested and the size of the biopsy.

Usually, the specimen was procured from the inner cheek of buccal mucosa; a few studies obtained samples from the interior of the lip [25, 26]. Concerning size, the range varied between 3–5 and 50 mm^2, but a few trials harvested biopsies up to 100–200 mm^2 [25, 27]. Independent of the biopsy site, a small sample of autologous oral mucosa is sufficient if an optimal culture method is used.

Culture Medium

The most common culture medium is a combination of DMEM/F12 (Dulbecco Modified Eagle's Medium with HAM F12), frequently used in a 1:1 ratio; some studies used 3:1 ratio. Keratinocyte growth medium (KGM), KBM-2 (serum-free keratinocyte growth medium), or supplemented hormonal epithelial medium (SHEM) has also been used. A critical parameter of culture media is the presence and origin of serum. Many studies used FBS or FCS (fetal bovine serum or fetal calf serum, respectively); however, the autologous serum (AS) from patients was explored in some studies to avoid any xeno-components. Moreover, Prabasawhat et al. used a serum-free medium [27]. The process is feasible by using a commercial clinical-grade serum, such as FBS or FCS, as individual batches can be strictly controlled and prove to be helpful in avoiding time-consuming and expensive setup of controls for each patient-derived serum sample. In addition, the risk of human cross-contamination (much more probable than animal-to-human) is reduced.

Feeder Layer

Except for a few studies [27, 28], a feeder layer was usually employed to support the growth of keratinocytes. Culture protocols frequently used gamma-irradiated or mitomycin C-treated 3T3 cells (murine fibroblast cell line). Sharma et al. performed an in vitro study comparing 3T3 to human autologous dermal-derived fibroblast feeder layer and found comparable performances [29]. Worth of note, it has been demonstrated that the use of mitomycin C to inactivate the feeder layer drastically reduces the long-term culture of normal human cells compared to gamma irradiation, owing to a significant decrease in cell metabolism [30].

Seeding

Another important difference among the culture methods is the type of seeding protocol used. Seeding is performed using single-cell suspensions obtained after enzymatic treatment or using an explant culture from the small pieces of the trimmed biopsy. The former technique is more common and allows for a more representative situation of the cell types constituting the biopsy, while the latter selects a subpopulation of highly migrating cells and has been adopted in only a few studies [28, 31].

Substrate

In many protocols, cells are grown over a substrate, which is often a denuded amniotic membrane (dAM, devoid of the epithelial layer) or a fibrin gel [21]. Kolli et al. used an intact amniotic membrane [28]. A temperature-responsive substrate made of poly(N-isopropylacrylamide) polymer (CellSeed®) was used in three studies [14, 15, 32]. The CAOMECS name is used when referring to this latter method. Kim and colleagues conducted a clinical study with a biomaterial-free cultured oral mucosal sheet [25•]. Some in vitro preclinical studies have also tested the viability of collagen-coated inserts [33]. To date, only a few substrates, such as fibrin polymer and amniotic membrane (in CLET), have been proven to be efficacious in a clinical setting after a long-term follow-up analysis [34].

AM and fibrin have been frequently used as carriers for COMET surgery. CAOMECS studies used a polyvinylidene fluoride membrane ring or a supporter [14, 15, 32], whereas other studies preferred the use of a filter paper ring or a support mesh [25, 35].

Culture Method

Lastly, other features that distinguish the culture methods of oral mucosal cells among the clinical trials are the days in culture and the choice of submerged or airlifted cultures. The total culture time of oral mucosal cells before transplantation was at least 1 week, with a maximum of 3 weeks. The

airlifted cultures were used in approximately two-thirds of the studies, whereas others maintained the cells in a submerged environment [13••]. It is worth noting that submerged culture allows the development of a confluent monolayer of the oral mucosal tissue and an optimal expansion of the stem cell compartment. This approach avoids the stressful and stemness-consuming process of airlift-forced differentiation.

Molecular Markers in Follow-Up Analysis

In biological models, molecular markers can be used as flags for specific activities in a defined location. These can be RNA-based markers (mRNAs and miRNAs), DNA-based markers (genomic-specific sequences of DNA and parts of chromosomes), and other molecules, such as amino acid–based markers, proteins, and their derivatives.

In the COMET technique, protein markers are usually employed both pre- and post-transplantation. Before transplantation, they are used to detect LSCD for highlighting the replacement of corneal cells with conjunctival cells in the cornea (see "Diagnosis" paragraph of this review). After surgery, the determination of molecular markers is important for follow-up analysis to estimate the replacement of the conjunctival *pannus* and to monitor the type of epithelium covering the corneal surface, as well as to understand the biological mechanism that drives corneal regeneration [16].

As shown in Table 1, of the 27 COMET clinical studies published between 2014 and 2020, only nine investigated the tissue phenotype in the transplanted eyes by molecular markers [25, 28, 31, 32, 37, 39, 40, 42, 47], while 18 did not [14, 15, 26, 27, 35–38, 41, 43–46, 48–52]. Several studies could not assess molecular markers using immunohistochemical analysis, because they did not perform a penetrating keratoplasty (PK) and, thus, did not obtain a corneal button specimen, or also because they stopped at mid-term follow-up analysis [37].

Methodologies

In the nine studies that were selected, specimens were embedded using OCT (optical cutting temperature) in five studies, FFPE (formalin-fixed paraffin-embedded) in three studies, and IC (impression cytology) in one study. Samples prepared using OCT and FFPE were analyzed using immunofluorescence (IF) and immunohistochemistry (IHC), respectively, whereas periodic acid–Schiff (PAS) staining was performed on samples prepared using both IC [40] and FFPE [31].

Limited Analyses of Failures

Regrettably, very few analyses have been performed on failed or partially failed cases [25, 39, 42], despite the importance of these data. Indeed, transplanted corneas that failed to improve visual acuity, transparency, and devascularization are highly relevant for understanding the biological pathways underlying the mechanisms of action.

Progenitor Markers

To predict the long-term stability of the transplant, corneal buttons are often analyzed for stem cell markers. The oral mucosa epithelium shares several progenitor markers with the cornea, such as p63 and Bmi1 [53, 54]. Indeed, p63, a stem cell marker of several epithelia, has been often investigated in patients who have undergone COMET [28, 31, 32, 41]. Chen et al. also analyzed the ABCG2 marker and p63, which were expressed in the cornea, oral mucosa, oral mucosal sheets, and corneal buttons after successful transplantations [42]. The oral mucosa stem cell marker p75 was found to be differentially expressed in the cornea and oral mucosa, although it was expressed in all patients [42, 55]; controversial results have been presented in the case report of Gaddipati et al. [31].

Cytokeratin Markers

Cytokeratins are structural cytoskeleton proteins in the form of intermediate filaments that are typical of epithelial cells [56]. They are the most frequently analyzed markers.

The corneal-specific keratins are K3 and K12; the conjunctival tissue expresses mainly K13, K19, and K7, although a few cells expressing these markers can also be found in the cornea [57]. The epithelium of the oral mucosa shares the expression of several ocular surface markers, such as K3 and K13, but not K12 [58]. Among the nine studies, including those that conducted immunophenotypical analyses, five detected K3-positive cells in successful cases, which could be related to the presence of both corneal and oral cells [28, 31, 37, 39, 42], although this was not investigated. In four studies, K13 was identified in the corneal button of successful patients but, as before, it could be referred to both oral mucosa and conjunctival cells [25, 37, 39, 42]. Indeed, K13 positivity was also found in failed patients in two reports, probably owing to the presence of conjunctival cells [25, 39]. K12 expression, which is unique in corneal cells, was found in successful cases of two studies and was negative in successful patients of three studies [25, 31, 39, 42]. In the former case, the success of the transplants can likely be attributed to the stimulation of the resident corneal stem cells producing a K12-positive epithelium, whereas in the latter, it can be associated with the engraftment of oral mucosa sheets. These hypotheses are supported by two observations: (i) K12 was negative in failed patients in the study by Nakamura of 2007 [39] and (ii) the study by Kim et al. showed positivity for K12 in a partial failure, indicating that the presence of few corneal cells was not sufficient for regeneration of the entire ocular surface. Indeed, the presence

Table 1 List of COMET clinical studies. Clinical trials and case reports performing COMET/CAOMECS procedures published from 2004 to 2020.

Author, year, journal (ref.)	N eyes/ N ptt.	Etiology (N eyes)	Success phenotype	Failure phenotype	Methodology	Vascularization assessment and related markers	Follow-up (average and range in months)
Nakamura *et al.*, 2004, Br J Ophthalmol. (41)	6/4	chemical burns (3), SJS (3)	-	-	-	Slit lamp observation	13.8 ± 2.9 (11-17)
Nishida *et al.*, 2004, N Engl J Med. (14)	4/4	SJS (1), OCP (3)	-	-	-	Not specified assessment of vascularization	14 ± 1 (13-15)
Inatomi *et al.*, 2006, Am J Ophtalmol. (36)	2/2	SJS (1), chemical burns (1)	K4+, K13+, K1-, K3+, K12-	-	IF on OCT	Not specified assessment of vascularization	32 (19-26)
Inatomi *et al.*, 2006, Am J Ophtalmol. (20)	15/12	SJS (7), chemical burns (5). 1 thermal injury (1), OCP (1)	-	-	-	Slit lamp observation	20 (up to 34)
Ang *et al.*, 2006, Arch Ophthalmol. (51)	10/10	SJS (7), OCP (1), chemical injury (1), thermal injury (1)	-	-	-	Slit lamp observation	12.6 ± 3.9 (up to 19)
Nakamura *et al*, 2007, Ophthalmology (40)	6/5	SJS (3), chemical burn (3)	K3+, K12-, K4+, K13+, Muc5ac-, ZO-1+, Col7+, Lam5+	K3-, K12-, K4+, K13+, Muc5ac+, ZO-1+, Col7+, Lam5+	IF on OCT	Slit lamp observation	16 (n.a.)
Satake *et al.*, 2008 Arch Ophthalmol. (37)	4/4	SJS (2), OCP (2)	PAS±	-	PAS staining on IC	Slit lamp observation	16 ± 7.6 (6-24)
Ma *et al.*, 2009, Eye (43)	5/5	Acute or chronic burns (5)	-	-	IVCM	Not specified assessment of vascularization	29.6 ± 3.6 (26-34)
Chen *et al.*, 2009, Invest Ophthalmol Vis Sci. (39)	4/4	Chemical burn (3), thermal burn (1)	K3+, K12-, K4+, K13+, K8-, Cx43+, MUC5AC-, Lam5+, pan-p63+, ABCG2+, p75+	-	IF on OCT	Not specified assessment of vascularization	31 ± 4 (27-35)
Takeda *et al.*, 2011, Am J Ophtalmol. (44)	3/3	Chemical burn (1), thermal burn (2)	-	-	-	-	30 ± 19.5 (11-50)
Nakamura *et al.*, 2011, Br J Ophthalmol. (45)	19/17	SJS (11), GVHD (1), burn (1), SCC (2), OCP (4)	-	-	-	Slit lamp observation	55 ± 17 (36-90)
	40/36	SJS (12), burns (11), OCP (17)	-	-	-	Slit lamp observation	25.5 (6-54.9)

Table 1 (continued)

Author, year, journal (ref.)	N eyes/ N ptt.	Etiology (N eyes)	Success phenotype	Failure phenotype	Methodology	Vascularization assessment and related markers	Follow-up (average and range in months)
Satake *et al.*, 2011 Ophthalmology (46)							
Priya *et al.*, 2011, Eye (47)	10/10	Chemical burn (9), SJS (1)	-	-	-	Not specified assessment of vascularization	18.6 (1-38)
Hirayama *et al.*, 2012, Invest Ophthalmol Vis Sci. (35)	32/32	Chemical injury (12), SJS (4), OCP (16)	-	-	-	Slit lamp biomicroscopic graduation	109.8 ± 47 146.6 ± 74.1
Burillon *et al.*, 2012 Invest Ophthalmol Vis Sci. (15)	26/25	Corneal burn (9), LS (4), An (3), NK (2), RK (3), CLH (3), severe trachoma (1), cystinosis (1), SGD (1), HCV (1)	-	-	-	Slit lamp observation (vascular pediculi and vessel activity)	12
Chen *et al.*, 2012, Invest Ophthalmol Vis Sci. (38)	6/6	Chemical injury (4), thermal burn (2)	K8-	K8± (partial failure)	IF on OCT	Histology (H&E staining) examination / FGF2, VEGF, COL18 (endostatin), PEDF, sFlt-1, TIMP3, TSP1, IL-1RA	36.7 ± 17
Sotozono *et al.*, 2013, Ophtalmology (53)	46/40	SJS (21), OCP (10), thermal or chemical injuries (7), idiopathic (3), radiation (1), GvHD (1), An (1), SCD (1), drug toxicity (1)	-	-	-	Not specified assessment of vascularization	28.7 (6.2-85.6)
Kocaba *et al.*, 2014, J Stem Cell Res Ther. (32)	23/22	Chemical burn (2), RK (3), Corneal burn (6), LS (3), An (2), SGD (1), NK (2), severe trachoma (1), CLH (1), congenital cataract (1), cystinosis corneal opacity (1), HCV (1)	K6+, pan-p63+	-	IHC on FFPE	Number of vascular pediculi and their activity level; histological analysis	28 (18-48)
Gaddipati *et al.*, 2014, Indian J Ophthalmol. (31)	1/1	Chemical burn (1)	K3+, K12+, K14-, K19+, p63+, p75+, PAX6+, Ki67+; PAS-	-	IHC and PAS staining on FFPE	Histology (H&E staining) examination; IHC / CD31, CD34	11
Kolli *et al.*, 2014, Stem Cell (28)	2/2	Chemical burn (2)	K3±, p63+, Ki67+, MUC5Ac-	-	IHC on FFPE	Not specified assessment of vascularization	16.5 (13-20)
Sotozono *et al.*, 2014, Arch Ophthalmol. (52)	10/9	SJS (3), thermal/chemical injury (5), OCP (2)	-	-	-	Not specified assessment of vascularization	23.3 (5.6-39.7)
Dobrowoloski *et al.*, 2015, Biomed Res Int. (48)	17/13	An (17)	-	-	-	Not specified assessment of vascularization	16 (14-18)
Prabhasawat *et al.*, 2016, Cell	20/18	SJS (10), chemical burn (7) others (3)	-	-	-	Slit lamp biomicroscopic graduation	31.9 ± 12.1 (8-50)

Table 1 (continued)

Author, year, journal (ref.)	N eyes/ N ptt.	Etiology (N eyes)	Success phenotype	Failure phenotype	Methodology	Vascularization assessment and related markers	Follow-up (average and range in months)
Tissue Bank (27)							
Baradaran-Rafii *et al*., 2017, Ocul Surf. (26)	14/14	Chemical burn (14)	-	-	-	Not specified assessment of vascularization	28.2 ± 8 (14-40)
Kim *et al*., 2018, Cornea (25)	8/8	SJS (6), OCP (1), chemical burn (1)	K1-, K4+, K8-, K12+, K13+, K19-	K1+, K4+, K8±, K12+, K13+, K19± (partial failure)	IF on OCT	Not specified assessment of vascularization	10.1 ± 4.8 (2.1-16.1)
Wang *et al*., 2019, Graefes Arch Clin Exp Ophthalmol. (49)	34/32	Chemical injury (16), thermal injury (18)	-	-	-	Slit lamp biomicroscopic graduation	16.1 ± 5.8
Lim *et al*., 2019, Med J Malaysia (50)	1/1	Chemical injury (1)	-	-	-	Not specified assessment of vascularization	10

SJS = Stevens-Johnson syndrome; OCP = ocular cicatricial pemphigoid (or Pseudo/OCP); LS = Lyell syndrome; An = congenital aniridia; NK = neuroparalytic keratitis; RK = rosacea keratitis; CLH = contact lens hypoxia; SGD = severe Groenouw dystrophy; HCV = hepatitis C; PAS = Periodic Acid Schiff; GvHD = graft-versus-host disease; SCD = Salzmann's corneal degeneration; SCC = squamous cell carcinoma; IF = immunofluorescence; IHC = immunohistochemistry; IC = impression cytology; IVCM = *in vivo* confocal microscopy; FFPE = formalin fixed paraffin embedded; OCT = optimal cutting temperature compound; n.a.= not available.

of conjunctival markers such as K13, K19, and K8 was detected [25•]. K19 expression was tested only in two studies and showed a controversial pattern [25, 31]. K4 expression was analyzed in four clinical trials but was found in both successful and failed cases [39, 42]. Thus, K4 is an uninformative marker because it is expressed in vivo in all three epithelial types that were investigated [58, 59]. K1, a marker of cornified epithelia such as skin, was absent in the successful cases in two studies [25, 37] but was marginally expressed in the superficial layers in a case of partial failure [37]. Its presence cannot be associated with a specific epithelium, because of its absence in in vivo oral mucosa and ocular superficial tissues; however, it can be associated with a pathologic condition which an abnormal cornified envelope develops.

Cytokeratins were further analyzed to determine the expression of K14 (in one study), K8, and K6 [25, 31, 42, 47]. In particular, K8 expression was studied by Chen et al. in 2009 and 2012 [42, 47]. They report that this keratin is expressed in all ocular surfaces but not in oral mucosa. Accordingly, the absence of K8 in COMET-treated corneal surface would highlight the presence of oral mucosa only. Subsequently, Kim and colleagues used the same marker in their study, but partial failures revealed only a mixed faint expression of K8 [25•].

The expression of K6 was studied in a clinical trial [32] and was found to be partially expressed in the four corneal buttons of successful cases. It is worth noting that K6 positivity has been associated only with the differentiated epithelium of the oral mucosa in a study by Sugiyama, in which a LSCD rabbit model was used [17]. However, it is important to consider that K6 is a mucosal and hyperproliferative epithelial marker, which can be expressed in pathological conditions and in the edge of wounded epithelia such as skin, which is otherwise negative [56]. In this context, the positivity of this ambiguous marker appears suggestive of a pathological environment.

Additional Markers

In the ocular surface, the conjunctiva is the only tissue composed of mucin-producing goblet cells, which secrete MUC5AC, a glycoprotein that is physiologically present in the conjunctival epithelium but not in the cornea. MUC5AC can support the diagnosis of conjunctivalization in LSCD [4••]. The lack of MUC5AC was used in three follow-up studies to prove the absence of the conjunctival epithelium in successful cases, and its presence was highlighted in failures [28, 39, 42]. The presence of goblet cells determined using PAS staining has also been adopted in two different studies, using either IC (partial presence of goblet cells) or FFPE (negative) [31, 45].

Concerning other markers, Nakamura et al. in 2007 expanded the immunofluorescence panel using ZO-1 (zonula occludens-1), COL7 (collagen 7), and LAM5 (laminin 5, also tested by Chen [42]) to respectively describe tight junctions and basal lamina. However, these analyses were not conclusive because the proposed markers were found in both successful and failed cases [39]. Chen et al. also studied the presence of Cx43 (connexin 43) to evaluate the existence of gap junctions [42]. Moreover, two studies have verified the presence of proliferating cells based on the Ki-67 marker [28, 31], but neither Cx43 nor Ki-67 were informative in distinguishing between the types of epithelia. Interestingly, Gaddipati et al. reported the use of the PAX6 nuclear marker, a master gene regulator of eye development [60], in their case report [31]. PAX6 could, therefore, be important to assess engraftment because it is expressed only in the epithelia of ocular surface (cornea and conjunctiva) and absent in the oral mucosa [61].

Need of Unique Markers for Oral Mucosa

Currently, the major concern is to find a marker that can distinguish oral mucosal tissue from ocular surface epithelia, to accurately characterize transplants in patients. We can assume that K12 and MUC5AC expressions (or PAS staining) are univocal for corneal and conjunctival tissues, respectively. K6 positivity (with the limitations mentioned above), negativity for K8 and PAX6 (present only on the epithelia of ocular surfaces), or a panel of markers differentially expressed in cornea and conjunctiva (e.g., K3 and K13) can be used to distinguish the three epithelia. A univocal marker for the oral mucosa would clarify the mechanism of action and help to drive therapeutic choices. The engraftment of the oral sheets would confirm a total anatomical LSCD and implies the presence of a non-corneal epithelium. The stimulation of resident corneal tissue implies the presence of residual limbal stem cells, despite the clinical phenotype of the eye, which are still able to interact with the surrounding tissues. Lastly, a mix of the two processes would be probable (Fig. 1b), or the transdifferentiation theory (discussed subsequently) could be considered.

Transdifferentiation Hypothesis

In order to evaluate these different hypotheses, other examples of human autologous oral mucosal transplantation have been investigated. The applications of autologous oral mucosa range from the field of urology, where the oral mucosa has been studied as a cell source for hypospadias defects [62], to the field of ophthalmology, as discussed in this review, and gastroenterology, for the treatment of esophageal ulcerations [63]. It is still debatable whether these cells are able to transdifferentiate into a different cell type when placed at ectopic sites [64]. This hypothesis has been described in vivo, but almost only in rat and mouse models [65–67]. In humans, a similar investigation would require markers univocally expressed by the "donor" site, but absent in the "receiving" tissue. Studies suggest an alternative option that cells retain

the memory of their site of origin. In 2006, Mavilio et al. reported the autologous ectopic transplantation of expanded genetically modified epidermal stem cells from the palm of the hand to the leg of a patient suffering from junctional epidermolysis bullosa (JEB). The follow-up analysis revealed that the expression pattern of the donor site was maintained, showing the expression of the palm-specific keratin 9, in the leg [68]. This study demonstrated that skin cells are able to retain their original transcriptional program and do not transdifferentiate or modify their skin differentiation patterns. Similarly, as highlighted in some studies constituting this review, COMET can be successfully used to replace corneal cells while retaining their original phenotype and expression profile [42]. However, this is a speculation and must be confirmed based on univocal markers by the oral mucosa.

Long-term follow-up analysis of the markers expressed in the ectopic site will be of value not only to understand the biologic mechanisms underlying corneal repair after COMET but also to determine the progress of repair after transplanting differentiated adult stem cells at ectopic sites.

Markers of Vascularization

The molecular mechanism by which the cornea becomes avascular and the blood vessels stop at the limbus zone is still not completely understood. In COMET patients, during follow-up, a peripheral vascularization of the cornea is common, while the central part remains without vessels. In failure cases, one of the major and more evident phenotypes was the revascularization of the central cornea. It seems that the oral mucosa, a vascularized tissue, is able to retain capillaries out of the center of the cornea to some extent when successfully engrafted.

Only two research groups have analyzed the angiogenesis, at the molecular level, the central cornea in subjects who underwent COMET [31, 42]. Other studies assessed vascularization only clinically, by using slit lamp biomicroscopy, and histologically using H&E staining and using IVCM.

In particular, Chen et al. [47] compared normal corneal epithelium with four COMET corneal buttons for eight different angiogenic-related factors. Among them, three anti-angiogenic proteins were absent in corneal buttons and positive in corneal epithelium: sFlt-1 (fms-like tyrosine kinase-1), TIMP-3 (tissue inhibitor of metalloproteinase-3), and TSP-1 (thrombospondin-1). In particular, TSP-1 was already found in a previous in vitro study in cultured cornea, while it was absent in cultivated oral mucosa [69]. Concerning the pro-angiogenic factors, Chen reported no modulation among the cornea, oral mucosa, and corneal buttons. This was the case for FGF-2 (fibroblast growth factor-2), VEGF (vascular endothelial growth factor), PEDF (pigment epithelium–derived factor), endostatin (or collagen XVIII, COL18), and IL-1ra (interleukin-1 receptor antagonist). On the contrary, Kanayama and colleagues found an increment of the FGF-2 marker in cultivated oral mucosa compared to cultivated cornea in vitro [70].

The second clinical report on the molecular analysis of vascularization in COMET corneas investigated the markers of blood vessel endothelial cells only, particularly CD31 and CD34 [31]. IHC confirmed the presence of capillaries beneath the graft.

Vascularization could be patient specific or also be related to the surgical approach, to the cell culture system, to the presence or absence of a specific scaffold, or to other variables. Thus, it should be studied in-depth in COMET patients during follow-up analysis with angiogenic-related markers, which are critical to shed lights on the physiology of the district. This knowledge will provide important clues on the secreted or cytosolic factors that could be administered or inhibited to maintain the transparency in the transplanted eyes.

Conclusions

This review provides an overview of COMET procedures by analyzing 27 clinical trials and case reports. Sixteen years after the first publication of the COMET procedure described by Nakamura and colleagues [71], there is a wide discrepancy among diagnostic parameters and follow-up analysis of the culture processes of the oral mucosa. In order to standardize protocols and outcomes, a global consensus has been recently published by The International LSCD Working Group, which provides comparability criteria between different studies, driving ophthalmologists and researchers to perform clinical trials.

In this review, a major focus has been given to molecular markers that are used to determine the success of a transplant and during the characterization of the follow-up of individuals after undergoing COMET. The usefulness of several markers, such as K12, PAX6, and MUC5AC, as well as PAS staining, has been highlighted. The role of the majority of the commonly expressed markers appears ambiguous. However, there is still a need for a univocal oral mucosal marker that can distinguish the three epithelia competing for re-epithelialization, namely, cornea, oral mucosa, and conjunctiva.

Lastly, much remains to be investigated regarding the role of angiogenesis and vascularization in the COMET procedure, as it is still unclear what could tip the balance between success and failure of the procedure.

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Compliance with Ethical Standards

Conflict of Interest Dr. Eustachio Attico and Dr. Giulia Galaverni declare no conflict of interest. Prof. Graziella Pellegrini is a member of the Board of Directors, R&D Director of Holostem Terapie Avanzate, and J-TEC consultant.

Human and Animal Rights All reported studies/experiments with human or animal subjects performed by the authors have been previously published and complied with all applicable ethical standards (including the Helsinki declaration and its amendments, institutional/national research committee standards, and international/national/institutional guidelines).

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- • Of importance
- •• Of major importance

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